*DOI:*
10.1007/s40429-015-0072-9

*Document Version*
Peer reviewed version

# DNA methylation, substance use and addiction: A systematic review of recent animal and human research from a developmental perspective

SELF-ARCHIVING VERSION

Charlotte A.M. Cecil, PhD[1], Esther Walton, PhD[1] & Essi Viding, PhD[2]

1. Department of Psychology, Institute of Psychiatry, Psychology and Neuroscience, King's College London, UK.
2. Division of Psychology and Language Sciences, University College London, UK.

**Key words:** Epigenetics, DNA methylation, addiction, alcohol, drug, review

**Citation**: Cecil, C.A.M., Walton, E. & Viding, E. (In Press). DNA methylation, substance use and addiction: A systematic review of recent animal and human research from a developmental perspective. *Current Addiction Reports.*

**Correspondence:** Charlotte Cecil, Department of Psychology, Institute of Psychiatry, Psychology & Neuroscience, King's College London, De Crespigny Park, London, SE5 8AF, UK. Tel: +44 (0)207 848 0389, E-mail: charlotte.cecil@kcl.ac.uk.

**Disclosures and acknowledgments:** All authors report no competing interests.

**Abstract**

Growing evidence points to the role of epigenetic mechanisms, including DNA methylation, in substance use and addiction. We conducted a systematic review of 47 recent (2012-15) animal and human studies that investigate DNA methylation and substance use/exposure, spanning preconception to adulthood. The majority of extant studies (i) focused on exposure during adulthood, (ii) examined the effects of alcohol use, (iii) employed a candidate gene approach and (iv) were cross-sectional. While studies generally support an association between substance use/exposure and DNA methylation, and also suggest that developmental context and timing matter, dearth of longitudinal data and low comparability across studies currently limits the conclusions that can be drawn. Future challenges and directions for the field are discussed.

## Introduction

Addiction to psychoactive substances (e.g. alcohol, illicit drugs) is a debilitating condition characterized by compulsive drug-seeking and repeated harmful use, despite adverse consequences [1]. Like other complex diseases, addiction results from both genetic and environmental factors, which combine to exert additive, evocative and interactive effects across the lifespan [2]. How such gene-environment associations operate at a molecular level, however, remains unclear. In recent years, epigenetic mechanisms have been proposed as a potential candidate, as they respond to both genetic and environmental influences [3, 4] and are thought to mediate vulnerability to disorders, including addiction [3, 4].

Epigenetic mechanisms, such as DNA methylation (DNAm) regulate when and where genes are expressed without changing the DNA sequence itself [5]. DNAm refers to the addition of a methyl group, primarily in the context of cytosine-guanine (CpG) dinucleotides. The genome contains in excess of 28 million CpG sites, around 10% of which cluster into CpG 'islands', close to gene promoter regions [6]. Methylated CpG islands impede transcription factors from accessing the DNA sequence. As such, DNAm is typically associated with decreased gene expression, although the functional role of methylation changes within genomic regions other than CpG islands (e.g. intergenic regions) remains unclear [7]. Importantly, DNAm is dynamic across the lifespan – although patterns are mitotically stable, which can lead to long-term alterations in gene activity, they also show a considerable degree of flexibility over time, enabling cells to respond to changing internal and external inputs [8].

A growing number of studies have begun to clarify the role of DNAm in substance abuse and addiction. Experimental studies in animals have led the way, documenting a number of important findings. First, substance use can alter DNAm – for example, repeated administration of substances (e.g. alcohol, cocaine) has been found to modify methylation patterns in the reward regions of the brain (e.g. striatum [9]). Second, DNAm contributes to the pathophysiology of addiction. Specifically, drug-induced methylation changes have been shown to influence the expression of genes involved in synaptic plasticity and memory consolidation, which in turn drive long-term neuroadaptations underlying the onset and persistence of addictive behaviours [4]. Third, animal studies have begun to shed light on the role of developmental context on DNAm and addiction risk. For example, alcohol intake during the first half of pregnancy has been found to alter epigenetic patterns in the developing embryo, leading to reduced fetal growth and congenital abnormalities similar to those observed in human fetal alcohol syndrome, as well as subsequent risk for addiction [10].

So far, studies in humans have provided initial support for animal findings, reporting methylomic differences between substance abusers and drug-free controls across a number of substances and tissue types [11, 9]. However, unlike animal studies that make it possible to experimentally manipulate the type, extent and timing of substance exposure, studies in humans have been primarily cross-sectional and correlational, making the causal links between epigenetic changes and subsequent addiction more problematic to draw.

The aim of this systematic review is three-fold: (i) to collate findings from recent animal and human research investigating the link between substance exposure, DNAm and addiction; (ii) to consider the relevance of timing of substance exposure, beginning in preconception through to adulthood; and (iii) to outline future directions for the field.

## Methods

### *Inclusion criteria*

We included studies that investigated associations between DNAm and substance use/exposure. In line with the journal's focus on current research, we only included articles published during the past three years (01-01-2012 to 31-02-2015). No restriction was applied regarding: (i) species (e.g. human, mouse), (ii) period of exposure (e.g. prenatal, adulthood), (iii) substance (e.g. alcohol, cocaine), (iv) tissue (e.g. blood, brain), (v) approach (e.g. candidate vs genome-wide), and (vi) design (e.g. cross-sectional vs longitudinal).

### *Search strategy*

PubMed and PsychInfo were searched for relevant studies written in English. Search terms were applied in MeSH or index terms, as well as text words. Included terms related to either (i) DNA methylation (e.g. methylat*; epigen*), or (ii) substance (e.g. substance use, abuse, dependence; drug; addiction; cocaine; heroin; cannabis; alcohol; opiate*; smoking; tobacco). 'Cancer' and 'medication' were specified as exclusion terms to avoid studies investigating DNAm in relation to clinical drugs.

### *Study selection*

Our search yielded 621 records, with 381 remaining after filtering out duplicates (see **Figure 1**). Titles and abstracts were screened and studies excluded if they were not empirical (e.g. reviews), focussed on epigenetic mechanisms other than DNAm (e.g. histone modifications) or examined

drugs other than the ones specified above (e.g. clinical drugs). Given that the majority of DNAm studies on tobacco use examined medical diseases (e.g. cancer) as opposed to addiction-relevant phenotypes, studies with tobacco were not included in the review. 61 studies were retained and their full-text articles were assessed for eligibility. 16 articles were removed due to the following reasons: (i) 6 did not include DNAm data; (ii) 6 did not report direct associations between DNAm and substance use/exposure; (iii) 2 did not include substance data; (iv) 1 was published before 2012; and (v) 1 was based on cell culture data. A total of 45 original reports were included in the systematic review.

## Results

### *Descriptive summary*

Study characteristics are summarized in **Table 1** (see also **Figure 2**). 24 the studies examined animal samples ($n_{rat}$ = 14 and $n_{mouse}$ = 10) and 21 examined humans. The majority of studies investigated substance exposure during adulthood (*n* = 33), focused on alcohol (*n* = 36), involved peripheral samples (*n* = 25), examined DNAm at a single time point (*n* = 40) and used a candidate gene approach (*n* = 26). The most common peripheral tissue examined was blood (*n* = 18), followed by liver, sperm, pancreas, saliva, placenta, kidney, intestine and colon. Most commonly examined central tissues were prefrontal cortex (*n* = 5) and hippocampus (*n* = 4), followed by nucleus accumbens, hypothalamus, amygdala, cerebellum, ventral tegmental area, striatum and neocortex. Below, we describe findings first in animals and then in humans, in order of developmental period of substance exposure.

### *Animal studies*

<u>Preconception</u>

Three candidate gene studies investigated parental alcohol use prior to conception. In the first, paternal consumption in mice related to decreased DNAm of *Bdnf* – implicated in stress response and neural development – in paternal sperm cells and offspring ventral tegmental area [12]. In the second, paternal alcohol consumption in rats was associated with increased *Pomc* methylation – another gene relevant in stress response –in both parental sperm and offspring hypothalamus, although findings were specific to the male germline [13]. In contrast, the third study [14] found that DNAm in *H19* CTCF binding sites - involved in imprinting mechanisms - was reduced in offspring tail blood but not paternal sperm cells.

Prenatal

Three studies from the same working group found that prenatal alcohol exposure associated with increased *Pomc* methylation in the rat hypothalamus, which in turn related to decreased gene expression [15, 16, 13]. These changes were maintained transgenerationally (up to three generations), but could be rescued by gestational choline supplementation. Epigenome-wide associations between prenatal alcohol exposure and DNAm in brain tissue from adult offspring were identified by one study, particularly within genetic pathways related to nervous system development (including the *Cdk5* signaling pathway) and neurological diseases, including the Alzheimer disease-linked gene *App* [17]. Another study found that in utero exposure to methamphetamine was associated with aberrant hippocampal DNAm in adolescent mice offspring [18]. Hypermethylated genetic pathways related to cerebral cortex GABAergic interneuron differentiation, while hypomethylated pathways related to embryonic development.

Neonatal

Two mouse studies investigated the effect of neonatal alcohol exposure on global methylation within the hippocampus and neocortex [19, 20]. While the first study reported a *reduction* in global methylation in response to acute alcohol exposure (8 and 24 hours post-exposure; [20]), the second study observed an *increase* in global methylation in the exposed group across both regions, which could be partially ameliorated by choline treatment [21]. Although of interest, it is important to note that neonatal substance exposure may be less relevant to human studies compared to other developmental periods, as it is relatively uncommon in humans.

Adulthood

This developmental period received by far the greatest research attention (71% of animal studies, $n = 17$) and was primarily examined in relation to alcohol exposure ($n = 11$). In global methylation studies, exposed mice were found to have lower DNAm in the cerebral cortex [21] but not in liver [22], although reductions were reported in global DNA hydroxymethylation (another type of DNA modification, characterized by the addition of a hydroxymethyl group). Findings from candidate gene studies further indicated that alcohol exposure in adulthood is associated with increased DNAm in multiple genes, including the serotonin receptor *Htr3a* in blood and hippocampal tissue [23], the sodium transporter *Slc5a6* gene in pancreatic tissue [24] and immune-function TLR-pathway genes in the liver [25]. Decreased DNAm was instead observed for the glutamate gene *Nr2b* in the prefrontal cortex [26] and *Bdnf* gene in motile sperm [12]. Tissue- and gene-specific DNAm alterations were identified by one study in folate-regulating genes [27]. Finally, no

associations were found in opioid-related genes *Pdny* and *Pnoc* in the rat amygdala [28], as well as imprinting-control genes *H19* and *Rasgfl* in mouse sperm [14].

Seven studies examined DNAm in relation to cocaine and/or opiate exposure. No associations with global methylation were reported in the corpus callosum of cocaine-exposed rats after 1 or 30 days of forced abstinence [29], as well as cocaine or heroin-exposed mice [30] – although a specific reduction in hydroxymethylation in the liver following cocaine administration was reported within the same sample [31]. Drug- and tissue-specific effects were also identified in [32], where global DNAm reductions were evident in the prefrontal cortex (but not nucleus accumbens) of mice exposed to cocaine (not heroin) – an effect that was reversible through repeated administration of methionine. With regards to candidate genes, increased *Drd2* receptor methylation was observed in the nucleus accumbens of rats exposed to glucocorticoids in utero [33]. This association was specific to morphine administration and reversed by L-dopa treatment. Also in the nucleus accumbens, repeated SAM pre-treatment was found to modify cocaine-induced methylation changes in the neuropeptides *Cck* and *Gal* as well as the glutamate transporter *Slc17a7* [34]. Pol Bodetto et al [35] reported that methylation of *Pp2cβ*, a gene involved in cellular function, was higher in the brain of cocaine-exposed rats vs controls. Finally, in a study investigating myelin-producing genes, reduced mean DNAm of *Sox10* was identified in the corpus callosum of cocaine-exposed rats, particularly after a period of forced abstinence [29]. None of the studies examined epigenome-wide alterations in response to adult substance exposure.

**Human studies**

Prenatal

Two studies examined DNAm in relation to prenatal alcohol exposure. Wilhelm-Benartzi et al. [36] found that maternal alcohol intake positively associated with global LINE-1 (but not AluYb8) methylation in placental tissue. One candidate gene study found that cord blood methylation of the developmental gene *ZAC1* positively correlated with prenatal maternal alcohol intake as well as associating with reduced fetal and postnatal weight [37].

Adolescence

Only one study examined adolescent substance use. Researching the impact of cannabis smoking on whole blood *COMT* methylation (important for neurotransmitter catalysis), van der Knaap et al [38] found no main effect of cannabis use. However, a significant methylation by genotype interaction

was identified, where Met/Met carriers with higher DNAm were least likely to be frequent cannabis users.

Adulthood

85% of studies in humans (*n* = 18) examined adult substance use, again focusing primarily on alcohol exposure (*n* = 16). One global methylation study found decreased DNAm in the blood of alcohol drinkers (Alu, not LINE-1 [39]). Results contrast those of increased global methylation identified in the frontal cortex of HIV+ methamphetamine users vs non users [40] as well as in the blood of methadone-substituted former opiate addicts, an effect which was also replicated in independent sample of opioid-treated patients [41].

Candidate gene studies focused mainly on genes involved in neural function, most likely guided by existing neurochemical data regarding addiction on animals and humans. Higher DNAm was observed in the blood of alcohol-dependent individuals within the *HTR3A* serotonin receptor gene [42] and *OPRM1* opioid receptor gene [43] – an association that was also identified in opiate addicts [41]. Lower DNAm of the leptin hormone (*LEP*) gene was instead identified in the blood of patients with stronger alcohol cravings [44]. No significant associations were reported between alcohol use and DNAm in a number of genes, including *PDNY* and *PNOC* opioid-related genes (blood; [42]), the serotonin transporter *5-HTT* in females exposed to trauma (alcohol, cannabis; [45]), the *DAT* dopamine transporter in blood [46] and the drug metabolism gene *UGT1A1* in human liver [47]. Of the candidate gene studies reviewed, two featured repeated measures of DNAm, comparing alcohol-dependent cases vs controls at baseline, day 7 and day 14 post-treatment admission. While the first found significant differences in DNAm of volume-regulating neuropeptides *AVP* and *ANP* both at baseline and between day 7 and 14 of withdrawal [48], the second [49] reported increased nerve growth factor (*NGF*) methylation in cases vs controls, but only between day 7 and 14.

All epigenome-wide investigations focussed on the effect of alcohol in blood. Generally, results were mixed, depending on sample characteristics and methods. In terms of specific genes, two EWAS studies confirmed associations with alcohol metabolism-related genes, including alcohol and aldehyde dehydrogenases (*ADH1A, ADH7, ALDH3B2, ALDH1A2*) and cytochrome P450 2A13 [50, 51]. In one study [52], the tumor suppressor gene *BLCAP* and *ABR* – involved in vestibular morphogenesis – were hypomethylated in heavy alcohol drinkers vs abstinent controls, suggesting one mechanism by which tumor risk may be higher in alcohol drinkers. In another study, alcohol-dependent discordant siblings showed hypomethylation of *SSTR4* – an important gene for hormonal

function – and hypermethylation of the GABA receptor gene *GABRP* [53]. Finally, two EWAS studies measured DNAm at multiple time points: before and after a 25-day treatment program [54], or a 12-year interim period [55]. While the former [54] found no significant differences pre-vs-post treatment, the latter [55] observed a general increase in methylation with alcohol consumption over a 12-year period, particularly in *CKM*, *PHOX2A* and *NPDC1*. With regards to wider biological pathways, EWAS studies indicated that the most common pathways that were hypermethylated in response to alcohol use were those related to G-protein mediated and GTPase signal transduction processes [55, 54, 51], whereas pathways associated with stimulus and stress responses, as well as immune and inflammatory processes, where likely to be hypomethylated [51]. Hypomethylation was also observed in long terminal repeat (LTR) regions of retrotransposons in the superior frontal cortex of post-mortem alcohol users [56]. Other important pathways related to apoptosis [55, 52, 54], metabolism [53], as well as GABA and dopamine systems [53, 40].

## Discussion

The aim of the present review was to summarize the latest animal and human research investigating the association between substance use, DNA methylation and addiction risk, spanning preconception to adulthood. Based on the 45 reports included, we may conclude that there is preliminary support for a link between substance exposure, DNAm and addiction. However, findings are often mixed and have limited comparability. In this section, we review key similarities and differences across studies, evaluate evidence for the importance of timing of substance exposure and outline future directions for the field.

### *Summary of study characteristics and findings*

The majority of studies across species focused on substance exposure during adulthood, examined the effects of alcohol, employed a candidate gene approach and were cross-sectional. One key difference related to tissue, with animal studies most often investigating brain samples and human studies examining DNAm in blood. Global methylation studies were more common in rodents, while epigenome-wide studies were more frequently carried out in humans. Although prospective longitudinal designs were more common in animal studies, the only four reports to include repeated DNAm measures were based on humans.

As a symptom of how young the field is, there are not sufficient data to assess how DNAm of specific genes relates to exposure to specific substances across developmental periods and tissue types. We do, however, highlight five genes that were investigated by multiple studies and,

promisingly, showed a consistent direction of associations. In three rodent studies from the same working group [15, 16, 13], increased methylation and decreased expression of *Pomc* – a gene implicated in stress response, metabolism and immune function – was observed in response to prenatal alcohol exposure across multiple tissues. These results highlight one mechanism through which fetal alcohol programming can occur, contributing to HPA axis dysregulation and increased addiction risk [16]. In two other studies, the opioid receptor mu 1 (*OPRM1*) – a gene important for mediating drug-induced activation of reward pathways – was hypermethylated in the blood of former opiate addicts [41] and alcohol-dependent individuals [43]. It was not possible to establish, however, whether higher methylation was a predisposing factor for addiction and/or a consequence of long-term substance use. Furthermore, hypermethylation of the serotonin receptor 3A (*HTR3A*) was identified in relation to alcohol exposure across both humans [42] and rodents [23]. Finally, a null association between alcohol exposure and methylation in the opioid signaling genes *PDNY* and *PNOC* was reported in human blood [42] and brain tissue in rats [28]. Despite these consistent findings, It is noteworthy that genes investigated by candidate studies did not typically converge with those identified by studies using hypothesis-free, epigenome-wide analyses. Instead, EWAS studies more often reported significant associations with drug metabolizing genes, as well as highlighting wider biological pathways linked to substance exposure, including signal transduction, inflammation, and apoptosis, in addition to stress response and neurotransmission. How these pathways specifically contribute to addiction, however, remains unclear.

Given the limited comparability across studies, we were not able to systematically assess the importance of developmental context in the relationship between substance use, DNAm and addiction risk. However, the studies reviewed did provide preliminary support for the relevance of timing of substance exposure on DNAm. For example, evidence from animal models demonstrated that substance exposure can influence DNAm even prior to conception, supporting the existence of transgenerational effects [12-14]. Studies also pointed to the prenatal period as a particularly sensitive developmental window. For example, in utero substance exposure influenced DNAm of developmental genes, which in turn affected postnatal outcomes (e.g. reduced postnatal weight [37]), although the relevance of these changes for addiction risk is yet to be characterized. It is important to note that the period between birth and adulthood received very little attention. In fact, none of the studies investigated childhood and only one examined adolescence – a key period of vulnerability for the development of substance use disorders [57].

*Current challenges for the field*

Despite considerable advances in epigenetic research, studies investigating the role of DNAm in substance use and addiction continue to face a number of key challenges [58, 11]. Firstly, *our knowledge of the methylome is still limited*. Because we know little about 'typical' methylation patterns in humans, it is difficult to establish when such patterns deviate to contribute to diseased states. This is complicated by the fact that DNAm patterns can vary across multiple factors, including tissue, cell-type, sex and age [59]. In general, the compilation of reference datasets will be important for providing a 'typical' benchmark against which to compare epigenetic findings. Knowledge is also limited regarding the relative contribution of genetic and environmental influences on observed methylation patterns, which will require the use of genetically-informative designs, such as twin studies and studies identifying methylation quantitative trait loci [60, 61]. More work will also be needed to determine the functional significance of identified DNAm changes at transcriptomic, metabolomic, proteomic and neural biological levels.

A second set of challenges relates to *research methodology*. Methods have varied widely across studies, including differences in pre-processing, quality control, genomic coverage, data analysis, choice of covariates and significance thresholds used for detecting effects. Together, these sources of variability have limited comparability across studies and complicated efforts to replicate findings – a necessary step for weeding out false-positives. The increased availability of standardized pipelines will considerably help in this respect [62]. Furthermore, the integration of discovery and replicate samples will become increasingly important, as was the case for genomic studies. More research will also be needed to determine what sample sizes are required to reach appropriate statistical power, although simulation-based studies are beginning to provide recommendations [63].

A third issue relates to *difficulties in establishing causal relationships* between substance use, DNAm and addiction. Most of the studies reviewed adopted a cross-sectional approach with DNAm data sampled at only one time point. Human studies, in particular, focussed primarily on adults who had already been exposed to substances. As such, it remains unclear whether DNAm can be a risk factor for, as well as a consequence of substance use, and how substance exposure and DNAm interrelate over time to influence addiction risk.

Below, we propose a number of ways in which future research may strengthen causal inferences and improve understanding of the role of DNAm in substance exposure and addiction.

*A proposed model for conducting research on DNA methylation, substance use and addiction*

Firstly, it will be important to capitalize on the strengths of animal models to clearly delineate the mechanisms linking substance exposure, DNAm and addiction. Systematic investigations will need to be conducted within a given substance, across multiple tissues, over developmental periods and in different strains. Importantly, it will be necessary to collect prospective, repeated measures of DNAm *pre*- and *post*- substance exposure, in order to (i) investigate whether pre-exposure DNAm predicts individual differences in drug-seeking behaviours, (ii) trace the timing and stability of DNAm changes following exposure, and (iii) clarify whether DNAm *mediates* the effect of substance use on the onset and persistence of addiction. The sampling of multiple tissues over time will also make it possible to establish cross-tissue variability and locate peripheral biomarkers that most closely resemble DNAm changes in neural networks underlying addiction. Furthermore, incorporating additional *omics* data, such as gene expression, serum levels, protein content and enzymatic activity, will be useful for clarifying the functional relevance of observed DNAm changes at multiple biological levels [e.g. 16, 13]. Importantly, the use of methyl-modifying agents (e.g. methionine, choline [15, 32]) will offer valuable opportunities for testing the reversibility of drug-induced DNAm changes, identifying whether certain developmental periods are more sensitive to intervention, and examining whether normalization of DNAm patterns parallel changes in addiction-relevant phenotypes. Finally, the availability of methylomic data in relation to different substances will enable to disentangle DNAm markers that are common to multiple substances (perhaps reflecting a general liability to addiction) as opposed to substance-specific markers.

The knowledge generated from animal research could then be used to inform the design of human studies and to map out the most promising DNAm markers for further investigation. This will require, however, the use of strategies to maximise cross-species comparability. For example, the use of data from epidemiological birth cohorts that feature repeated measures of DNA [e.g. 64], could allow researchers to examine whether pre- vs post-exposure DNAm changes identified in longitudinal animal studies extend to humans. Furthermore, analytic methods that make it possible to integrate repeated measures of environmental exposure (e.g. substance use), DNAm and phenotypic outcomes (e.g. addiction) – such as structural equation modelling – will be particularly useful for tracing longitudinal associations and for testing mediation hypotheses [65]. The development of advanced causal inference methods, such as two-step epigenetic Mendelian Randomization [66,67], may also show promise for testing causal pathways documented in animals. As discussed above, the inclusion of additional biological markers (e.g. serum levels) will be necessary for establishing the functional relevance of identified DNAm markers. Furthermore,

given the scarce availability of central tissues in human research (i.e. post-mortem), it will be important in future to investigate whether peripheral DNAm markers can be related to in vivo structural and functional brain data (e.g. striatal activity when viewing addiction-related cues). Finally, increased use of approaches that capitalize on co-methylation patterns between CpG sites, such as regional or network-based approaches, will be important for reducing multiple testing and increasing power to detect effects in humans, enabling to move beyond individual methylation sites towards wider biological systems [68].

*Conclusions*

DNA methylation is emerging as an important molecular mechanism mediating substance use response and addiction risk. However, the limited understanding of the epigenome, heterogeneity across studies, a reliance on cross-sectional designs and lack of replications make it difficult to interpret the relevance of the extant data for mechanisms of addiction. Rapid developments in knowledge, methodology and research designs will offer exciting opportunities for delineating the role of DNAm in the pathophysiology of addiction, as well as testing its potential clinical utility as an exposure indicator, disease biomarker and therapeutic target.

**References**

1. McQuown SC, Wood MA. Epigenetic regulation in substance use disorders. Current psychiatry reports. 2010;12(2):145-53. doi:10.1007/s11920-010-0099-5.
2. Kendler KS, Chen X, Dick D, Maes H, Gillespie N, Neale MC et al. Recent advances in the genetic epidemiology and molecular genetics of substance use disorders. Nature neuroscience. 2012;15(2):181-9. doi:10.1038/nn.3018.
3. Tsankova N, Renthal W, Kumar A, Nestler EJ. Epigenetic regulation in psychiatric disorders. Nature reviews Neuroscience. 2007;8(5):355-67. doi:10.1038/nrn2132.
4. Nestler EJ. Epigenetic mechanisms of drug addiction. Neuropharmacology. 2014;76 Pt B:259-68. doi:10.1016/j.neuropharm.2013.04.004.
5. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature genetics. 2003;33 Suppl:245-54. doi:10.1038/ng1089.
6. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M et al. DNA methylation profiling of human chromosomes 6, 20 and 22. Nature genetics. 2006;38(12):1378-85. doi:10.1038/ng1909.
7. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nature reviews Genetics. 2012;13(7):484-92. doi:10.1038/nrg3230.
8. Alabert C, Groth A. Chromatin replication and epigenome maintenance. Nature reviews Molecular cell biology. 2012;13(3):153-67. doi:10.1038/nrm3288.
9. Wong CC, Mill J, Fernandes C. Drugs and addiction: an introduction to epigenetics. Addiction (Abingdon, England). 2011;106(3):480-9. doi:10.1111/j.1360-0443.2010.03321.x.
10. Kaminen-Ahola N, Ahola A, Maga M, Mallitt KA, Fahey P, Cox TC et al. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. PLoS Genet. 2010;6(1):e1000811. doi:10.1371/journal.pgen.1000811.
11. Harlaar N, Hutchison KE. Alcohol and the methylome: design and analysis considerations for research using human samples. Drug and alcohol dependence. 2013;133(2):305-16. doi:10.1016/j.drugalcdep.2013.07.026.
12. Finegersh A, Homanics GE. Paternal alcohol exposure reduces alcohol drinking and increases behavioral sensitivity to alcohol selectively in male offspring. PloS one. 2014;9(6):e99078. doi:10.1371/journal.pone.0099078.

13. Govorko D, Bekdash RA, Zhang C, Sarkar DK. Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. Biol Psychiatry. 2012;72(5):378-88. doi:10.1016/j.biopsych.2012.04.006.
14. Knezovich JG, Ramsay M. The effect of preconception paternal alcohol exposure on epigenetic remodeling of the h19 and rasgrf1 imprinting control regions in mouse offspring. Frontiers in genetics. 2012;3:10. doi:10.3389/fgene.2012.00010.
15. Bekdash RA, Zhang C, Sarkar DK. Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and proopiomelanocortin (POMC) gene expression in beta-endorphin-producing POMC neurons of the hypothalamus. Alcoholism, clinical and experimental research. 2013;37(7):1133-42. doi:10.1111/acer.12082.
16. Gangisetty O, Bekdash R, Maglakelidze G, Sarkar DK. Fetal alcohol exposure alters proopiomelanocortin gene expression and hypothalamic-pituitary-adrenal axis function via increasing MeCP2 expression in the hypothalamus. PloS one. 2014;9(11):e113228. doi:10.1371/journal.pone.0113228.
17. Laufer BI, Mantha K, Kleiber ML, Diehl EJ, Addison SM, Singh SM. Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. Disease models & mechanisms. 2013;6(4):977-92. doi:10.1242/dmm.010975.
18. Itzhak Y, Ergui I, Young JI. Long-term parental methamphetamine exposure of mice influences behavior and hippocampal DNA methylation of the offspring. Molecular psychiatry. 2015;20(2):232-9. doi:10.1038/mp.2014.7.
19. Nagre NN, Subbanna S, Shivakumar M, Psychoyos D, Basavarajappa BS. CB1-receptor knockout neonatal mice are protected against ethanol-induced impairments of DNMT1, DNMT3A, and DNA methylation. Journal of neurochemistry. 2015;132(4):429-42. doi:10.1111/jnc.13006.
20. Otero NK, Thomas JD, Saski CA, Xia X, Kelly SJ. Choline supplementation and DNA methylation in the hippocampus and prefrontal cortex of rats exposed to alcohol during development. Alcoholism, clinical and experimental research. 2012;36(10):1701-9. doi:10.1111/j.1530-0277.2012.01784.x.
21. Fowler AK, Hewetson A, Agrawal RG, Dagda M, Dagda R, Moaddel R et al. Alcohol-induced one-carbon metabolism impairment promotes dysfunction of DNA base excision repair in adult brain. The Journal of biological chemistry. 2012;287(52):43533-42. doi:10.1074/jbc.M112.401497.

22. Tammen SA, Dolnikowski GG, Ausman LM, Liu Z, Sauer J, Friso S et al. Aging and alcohol interact to alter hepatic DNA hydroxymethylation. Alcoholism, clinical and experimental research. 2014;38(8):2178-85. doi:10.1111/acer.12477.

23. Barker JM, Zhang Y, Wang F, Taylor JR, Zhang H. Ethanol-induced Htr3a promoter methylation changes in mouse blood and brain. Alcoholism, clinical and experimental research. 2013;37 Suppl 1:E101-7. doi:10.1111/j.1530-0277.2012.01906.x.

24. Srinivasan P, Kapadia R, Biswas A, Said HM. Chronic alcohol exposure inhibits biotin uptake by pancreatic acinar cells: possible involvement of epigenetic mechanisms. American journal of physiology Gastrointestinal and liver physiology. 2014;307(9):G941-9. doi:10.1152/ajpgi.00278.2014.

25. Khachatoorian R, Dawson D, Maloney EM, Wang J, French BA, French SW et al. SAMe treatment prevents the ethanol-induced epigenetic alterations of genes in the Toll-like receptor pathway. Experimental and molecular pathology. 2013;94(1):243-6. doi:10.1016/j.yexmp.2012.09.024.

26. Qiang M, Li JG, Denny AD, Yao JM, Lieu M, Zhang K et al. Epigenetic mechanisms are involved in the regulation of ethanol consumption in mice. Int J Neuropsychopharmacol. 2014;18(2). doi:10.1093/ijnp/pyu072.

27. Wani NA, Hamid A, Kaur J. Alcohol-associated folate disturbances result in altered methylation of folate-regulating genes. Molecular and cellular biochemistry. 2012;363(1-2):157-66. doi:10.1007/s11010-011-1168-8.

28. D'Addario C, Caputi FF, Ekstrom TJ, Di Benedetto M, Maccarrone M, Romualdi P et al. Ethanol induces epigenetic modulation of prodynorphin and pronociceptin gene expression in the rat amygdala complex. Journal of molecular neuroscience : MN. 2013;49(2):312-9. doi:10.1007/s12031-012-9829-y.

29. Nielsen DA, Huang W, Hamon SC, Maili L, Witkin BM, Fox RG et al. Forced Abstinence from Cocaine Self-Administration is Associated with DNA Methylation Changes in Myelin Genes in the Corpus Callosum: a Preliminary Study. Frontiers in psychiatry. 2012;3:60. doi:10.3389/fpsyt.2012.00060.

30. Fragou D, Zanos P, Kouidou S, Njau S, Kitchen I, Bailey A et al. Effect of chronic heroin and cocaine administration on global DNA methylation in brain and liver. Toxicology letters. 2013;218(3):260-5. doi:10.1016/j.toxlet.2013.01.022.

31. Chao MR, Fragou D, Zanos P, Hu CW, Bailey A, Kouidou S et al. Epigenetically modified nucleotides in chronic heroin and cocaine treated mice. Toxicology letters. 2014;229(3):451-7. doi:10.1016/j.toxlet.2014.07.023.

32. Tian W, Zhao M, Li M, Song T, Zhang M, Quan L et al. Reversal of cocaine-conditioned place preference through methyl supplementation in mice: altering global DNA methylation in the prefrontal cortex. PloS one. 2012;7(3):e33435. doi:10.1371/journal.pone.0033435.

33. Rodrigues AJ, Leao P, Pego JM, Cardona D, Carvalho MM, Oliveira M et al. Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids. Molecular psychiatry. 2012;17(12):1295-305. doi:10.1038/mp.2011.126.

34. Anier K, Malinovskaja K, Pruus K, Aonurm-Helm A, Zharkovsky A, Kalda A. Maternal separation is associated with DNA methylation and behavioural changes in adult rats. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2014;24(3):459-68. doi:10.1016/j.euroneuro.2013.07.012.

35. Pol Bodetto S, Carouge D, Fonteneau M, Dietrich JB, Zwiller J, Anglard P. Cocaine represses protein phosphatase-1Cbeta through DNA methylation and Methyl-CpG Binding Protein-2 recruitment in adult rat brain. Neuropharmacology. 2013;73:31-40. doi:10.1016/j.neuropharm.2013.05.005.

36. Wilhelm-Benartzi CS, Houseman EA, Maccani MA, Poage GM, Koestler DC, Langevin SM et al. In utero exposures, infant growth, and DNA methylation of repetitive elements and developmentally related genes in human placenta. Environmental health perspectives. 2012;120(2):296-302. doi:10.1289/ehp.1103927.

37. Azzi S, Sas TC, Koudou Y, Le Bouc Y, Souberbielle JC, Dargent-Molina P et al. Degree of methylation of ZAC1 (PLAGL1) is associated with prenatal and post-natal growth in healthy infants of the EDEN mother child cohort. Epigenetics : official journal of the DNA Methylation Society. 2014;9(3):338-45. doi:10.4161/epi.27387.

38. van der Knaap LJ, Schaefer JM, Franken IH, Verhulst FC, van Oort FV, Riese H. Catechol-O-methyltransferase gene methylation and substance use in adolescents: the TRAILS study. Genes, brain, and behavior. 2014;13(7):618-25. doi:10.1111/gbb.12147.

39. Zhu ZZ, Hou L, Bollati V, Tarantini L, Marinelli B, Cantone L et al. Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. International journal of epidemiology. 2012;41(1):126-39. doi:10.1093/ije/dyq154.

40. Desplats P, Dumaop W, Cronin P, Gianella S, Woods S, Letendre S et al. Epigenetic alterations in the brain associated with HIV-1 infection and methamphetamine dependence. PloS one. 2014;9(7):e102555. doi:10.1371/journal.pone.0102555.

41. Doehring A, Oertel BG, Sittl R, Lotsch J. Chronic opioid use is associated with increased DNA methylation correlating with increased clinical pain. Pain. 2013;154(1):15-23. doi:10.1016/j.pain.2012.06.011.

42. Zhang H, Herman AI, Kranzler HR, Anton RF, Zhao H, Zheng W et al. Array-based profiling of DNA methylation changes associated with alcohol dependence. Alcoholism, clinical and experimental research. 2013;37 Suppl 1:E108-15. doi:10.1111/j.1530-0277.2012.01928.x.
43. Zhang H, Herman AI, Kranzler HR, Anton RF, Simen AA, Gelernter J. Hypermethylation of OPRM1 promoter region in European Americans with alcohol dependence. J Hum Genet. 2012;57(10):670-5. doi:10.1038/jhg.2012.98.
44. Hillemacher T, Weinland C, Lenz B, Kraus T, Heberlein A, Glahn A et al. DNA methylation of the LEP gene is associated with craving during alcohol withdrawal. Psychoneuroendocrinology. 2015;51:371-7. doi:10.1016/j.psyneuen.2014.10.014.
45. Beach SR, Brody GH, Lei MK, Gibbons FX, Gerrard M, Simons RL et al. Impact of child sex abuse on adult psychopathology: a genetically and epigenetically informed investigation. Journal of family psychology : JFP : journal of the Division of Family Psychology of the American Psychological Association (Division 43). 2013;27(1):3-11. doi:10.1037/a0031459.
46. Nieratschker V, Grosshans M, Frank J, Strohmaier J, von der Goltz C, El-Maarri O et al. Epigenetic alteration of the dopamine transporter gene in alcohol-dependent patients is associated with age. Addiction biology. 2014;19(2):305-11. doi:10.1111/j.1369-1600.2012.00459.x.
47. Yasar U, Greenblatt DJ, Guillemette C, Court MH. Evidence for regulation of UDP-glucuronosyltransferase (UGT) 1A1 protein expression and activity via DNA methylation in healthy human livers. The Journal of pharmacy and pharmacology. 2013;65(6):874-83. doi:10.1111/jphp.12053.
48. Glahn A, Riera Knorrenschild R, Rhein M, Haschemi Nassab M, Groschl M, Heberlein A et al. Alcohol-induced changes in methylation status of individual CpG sites, and serum levels of vasopressin and atrial natriuretic peptide in alcohol-dependent patients during detoxification treatment. European addiction research. 2014;20(3):143-50. doi:10.1159/000357473.
49. Heberlein A, Muschler M, Frieling H, Behr M, Eberlein C, Wilhelm J et al. Epigenetic down regulation of nerve growth factor during alcohol withdrawal. Addiction biology. 2013;18(3):508-10. doi:10.1111/j.1369-1600.2010.00307.x.
50. Harlaar N, Bryan AD, Thayer RE, Karoly HC, Oien N, Hutchison KE. Methylation of a CpG site near the ALDH1A2 gene is associated with loss of control over drinking and related phenotypes. Alcoholism, clinical and experimental research. 2014;38(3):713-21. doi:10.1111/acer.12312.
51. Zhang R, Miao Q, Wang C, Zhao R, Li W, Haile CN et al. Genome-wide DNA methylation analysis in alcohol dependence. Addiction biology. 2013;18(2):392-403. doi:10.1111/adb.12037.

52. Philibert RA, Plume JM, Gibbons FX, Brody GH, Beach SR. The impact of recent alcohol use on genome wide DNA methylation signatures. Frontiers in genetics. 2012;3:54. doi:10.3389/fgene.2012.00054.

53. Zhao R, Zhang R, Li W, Liao Y, Tang J, Miao Q et al. Genome-wide DNA methylation patterns in discordant sib pairs with alcohol dependence. Asia-Pacific psychiatry : official journal of the Pacific Rim College of Psychiatrists. 2013;5(1):39-50. doi:10.1111/appy.12010.

54. Philibert RA, Penaluna B, White T, Shires S, Gunter T, Liesveld J et al. A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs. Epigenetics : official journal of the DNA Methylation Society. 2014;9(9):1212-9. doi:10.4161/epi.32252.

55. Weng JT, Wu LS, Lee CS, Hsu PW, Cheng AT. Integrative epigenetic profiling analysis identifies DNA methylation changes associated with chronic alcohol consumption. Computers in biology and medicine. 2014. doi:10.1016/j.compbiomed.2014.12.003.

56. Ponomarev I, Wang S, Zhang L, Harris RA, Mayfield RD. Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012;32(5):1884-97. doi:10.1523/jneurosci.3136-11.2012.

57. Crews F, He J, Hodge C. Adolescent cortical development: a critical period of vulnerability for addiction. Pharmacology, biochemistry, and behavior. 2007;86(2):189-99. doi:10.1016/j.pbb.2006.12.001.

58. Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. Nature reviews Genetics. 2013;14(8):585-94. doi:10.1038/nrg3405.

59. Liang L, Cookson WOC. Grasping nettles: cellular heterogeneity and other confounders in epigenome-wide association studies. Human Molecular Genetics. 2014;23(R1):R83-R8. doi:10.1093/hmg/ddu284.

60. Bell JT, Pai AA, Pickrell JK, Gaffney DJ, Pique-Regi R, Degner JF et al. DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome biology. 2011;12(1):R10-R. doi:10.1186/gb-2011-12-1-r10.

61. Bell JT, Saffery R. The value of twins in epigenetic epidemiology. International journal of epidemiology. 2012. doi:10.1093/ije/dyr179.

62. Morris TJ, Beck S. Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data. Methods (San Diego, Calif). 2015;72:3-8. doi:10.1016/j.ymeth.2014.08.011.

63. Tsai P-C, Bell JT. Power and sample size estimation for epigenome-wide association scans to detect differential DNA methylation. International journal of epidemiology. 2015. doi:10.1093/ije/dyv041.

64. Relton CL, Gaunt T, McArdle W, Ho K, Duggirala A, Shihab H et al. Data Resource Profile: Accessible Resource for Integrated Epigenomic Studies (ARIES). International journal of epidemiology. 2015. doi:10.1093/ije/dyv072.

65. Cecil CA, Lysenko LJ, Jaffee SR, Pingault JB, Smith RG, Relton CL et al. Environmental risk, Oxytocin Receptor Gene (OXTR) methylation and youth callous-unemotional traits: a 13-year longitudinal study. Molecular psychiatry. 2014;19(10):1071-7. doi:10.1038/mp.2014.95.

66. Relton CL, Davey Smith G. Two-step epigenetic Mendelian randomization: a strategy for establishing the causal role of epigenetic processes in pathways to disease. International journal of epidemiology. 2012;41(1):161-76. doi:10.1093/ije/dyr233.

67. Pingault JB, Cecil CAM, Murray J, Munafò MR, Viding E. Causal inference in psychopathology: A systematic review of Mendelian randomisation studies aiming to identify environmental risk factors for psychopathology. Psychopathology Review. In Press.

68. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC bioinformatics. 2008;9:559. doi:10.1186/1471-2105-9-559.

**Figure legends**

**Figure 1.** PRISMA flowchart detailing the filtering steps undertaken to select studies

**Figure 2.** Flowchart of study characteristics**.** *N.B.* The same study may fit into multiple categories. Grey-shaded boxes represent developmental periods that have not been investigated. For each level (1-5) boxes are shaded according to study frequency (e.g. the least frequently investigated tissue is represented by lighter shading, while the most commonly investigated tissue is represented by darker shading). Boxes marked with an asterisk indicate presence of longitudinal studies with repeated measures of DNA methylation (here, boxes indicate the number of longitudinal studies out of the total number of studies). Abbreviations: Meth = methamphetamine; C = central tissue (brain); P = peripheral tissue; Ca = candidate gene; Ew = epigenome-wide; Gl = global.

**Figure 1.** PRISMA flowchart detailing the filtering steps undertaken to select studies

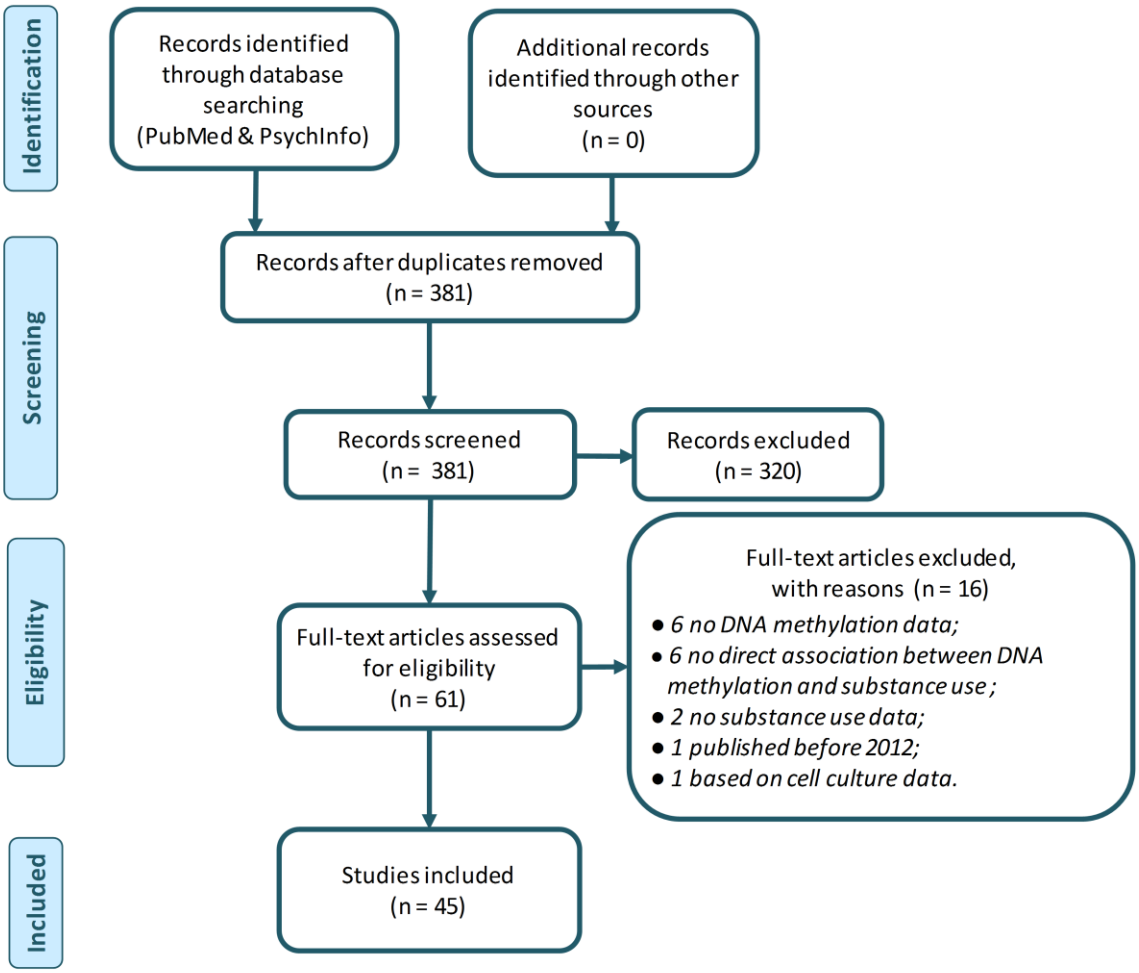

**Table 1.** Summary of study characteristics

| **Summary Table (*N = 45*)** | |
|---|---|
| **Developmental period** | |
| *Preconception* | 3 |
| *Prenatal* | 6 |
| *Neonatal* | 3 |
| *Childhood* | 0 |
| *Adolescence* | 1 |
| *Adulthood* | 33 |
| **Time points (DNAm)** | |
| *1 TP* | 40 |
| *2 TP* | 3 |
| *3 TP* | 2 |
| **Approach** | |
| *Candidate gene/s* | 26 |
| *Global DNAm* | 12 |
| *EWAS* | 9 |
| **Species** | |
| *Animal* | 24 |
| *Human* | 21 |
| **Tissue** | |
| *Peripheral* | 25 |
| *Central* | 21 |
| **Substance** | |
| *Alcohol* | 36 |
| *Cocaine* | 6 |
| *Cannabis* | 6 |
| *Opiates* | 3 |
| *Methamphetamine* | 2 |

*N.B.* The total number of studies for each characteristic may exceed 45 due to the presence of studies fitting multiple domains. To clarify, global methylation studies examine proxy markers of 'global' DNAm, using repetitive elements such as Alu and Line-1 (comprising of 11-17% of the genome). Candidate studies focus on DNAm in individual, preselected genes (typically one) based on a priori hypotheses, while epigenome-wide studies (EWAS) are hypothesis-free and investigate thousands of DNAm markers across the genome.

**Figure 2**. Flowchart of study characteristics

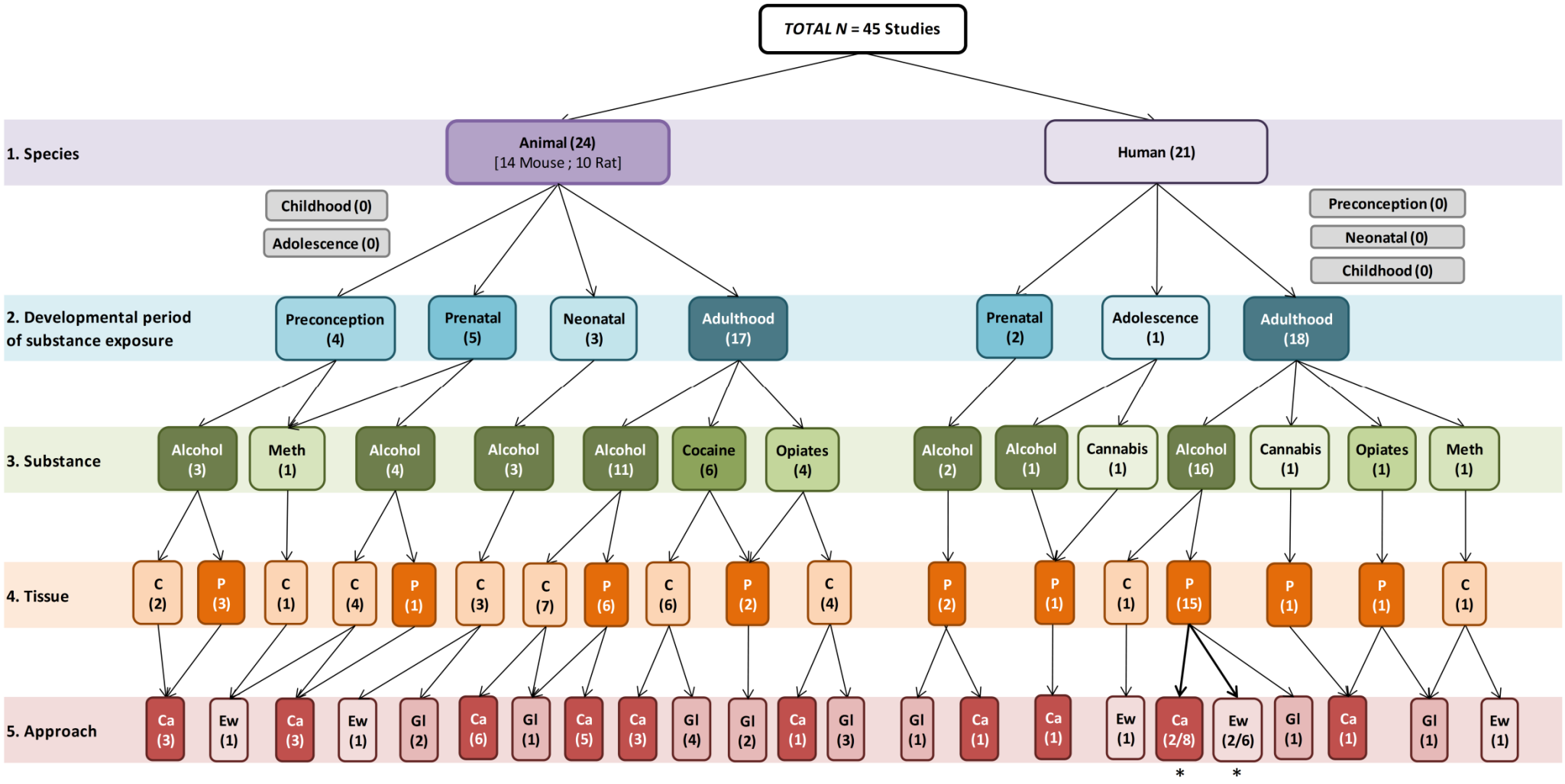

**Table 2.** Summary of study findings

| | Reference | Species | Period of substance exposure | DNAm *N* time points | Substance | Tissue | Approach | Gene | Sample | Findings |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Knezovich & Ramsay (2012)* | Animal (mouse) | Pre-conception (F2); Adulthood (F1) | 1 | Alcohol | Sperm (F1); tail blood (F2) | Candidate | *H19,Rasgfl* | F1: Male mice, 8 alcohol-treated vs 7 controls. F2: offspring from alcohol-treated (n = 17 ) vs controls sires (n = 29). | No significant difference in sperm methylation between alcohol-treated sires vs controls. *H19* CTCF binding sites were significantly hypomethylated in alcohol-sired offspring compared to controls, which in turn associated with age restricted growth retardation. |
| 2 | *Finegersh & Homanics (2014)* | Animal (mouse) | Pre-conception (F2); Adulthood (F1) | 1 | Alcohol | Sperm (F1); VTA; PFC (F2) | Candidate | *Bdnf* | F1: Male mice, 25 exposed to ethanol vapour vs 27 controls. F2: offspring from alcohol-treated (n = 123; 45% F) vs control sires (n = 104; 50% F). | Paternal ethanol exposure associates with decreased *Bdnf* promoter methylation in motile sperm as well as VTA of ethanol-sired male and female offspring. |
| 3 | *Govorko et al. (2012)* | Animal (rat) | Preconception; prenatal | 1 | Alcohol | Hypothalamus (ARC, PVN); blood (mononuclear cells); sperm | Candidate | *Pomc* | Male and female rats prenatally (GD 7-21) exposed to either an alcohol-liquid diet, a control-liquid diet or rat chow (n = 8/group). Also include F2 and F3 generation (n = 6-10/group). | Prenatal alcohol exposure associated with increased *Pomc* methylation in POMC neurons and sperm. Suppression of methylation normalized expression levels and function. Alcohol-induced methylation alterations in exposed F1 offspring transmitted to F2 and F3 male (but not female) germline. |
| 4 | *Itzhak et al. (2015)* | Animal (mouse) | Preconception; prenatal | 1 | Meth | Hippocampus | EWAS | - | 60 offspring of methamphetamine-exposed (pre- and postconception) and 62 offspring of control mice. DNAm collected for a subset of female mice (PD40–PD45). DNAm platform: MeDIP; Roche NimbleGen 385k. | 62 and 35 promoter regions, in which DNA methylation was elevated and reduced, respectively, in the F1 mice due to in utero methamphetamine exposure. Enriched pathways included 'cerebral cortex GABAergic interneuron differentiation' for hypermethylated sites and 'embryonic development' for hypomethylated sites. |
| 5 | *Bekdash et al. (2013)* | Animal (rat) | Prenatal | 1 | Alcohol | Hypothalamus (mediobasal) | Candidate | *Pomc* | Male rats prenatally (GD 7-21) exposed to an alcohol-liquid diet or a control diet, with or without choline (n = 5/group). | Prenatal alcohol exposure associated with increased *Pomc* methylation and decreased expression in the hypothalamus. Gestational choline normalized alcohol-induced effects on *Pomc* methylation, expression and stress-axis function. |
| 6 | *Gangisetty et al. (2014)* | Animal (rat) | Prenatal | 1 | Alcohol | Hypothalamus | Candidate | *Pomc* | Male rats exposed to alcohol prenatally (GD 7-21) vs controls. | Prenatal alcohol exposure associated with increased *Pomc* methylation and decreased expression in the hypothalamus. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | *Laufer et al. (2013)* | Animal (mouse) | Prenatal; neonatal | 1 | Alcohol | Whole brain | EWAS | - | Adult male mice (PD70), 6 alcohol-exposed (cases) and 6 matched controls. | 6660 promoter regions differentially methylated. Enriched pathways related to cell death and nervous system development. Network analysis indicated that the 'Behavior, Neurological Disease, and Psychological Disorders' network was the most significantly affected network, with *App* identified as a hub gene. |
| 8 | *Nagre et al. (2015)* | Animal (mouse) | Neonatal | 1 | Alcohol | Neocortex; hippocampus | Global | - | Neonatal (PD7) mice treated with ethanol (n=10) or saline (n=10);gender-matched. | Global DNA methylation was reduced after ethanol exposure (8 and 24 h) compared to saline treatment (0 h) in the hippocampus and neocortex. |
| 9 | *Otero et al. (2012)* | Animal (rat) | Neonatal | 1 | Alcohol | Hippocampus; PFC | Global | - | 28 choline-treated (13 ethanol/7 intubation control/8 non-treated control)+ 64 non-choline-treated mice (16ET/8IC/8NC); age- and sex-matched. | Ethanol treatment associated with hypermethylation in the prefrontal and hippocampal region and this was partially ameliorated by choline treatment. |
| 10 | *Fowler et al. (2012)* | Animal (mouse) | Adulthood | 1 | Alcohol | Cerebral cortex | Global | - | 10-12 week old male mice. | Long term but not short term ethanol exposure leads to OCM impairment seen from a significant decrease in global DNA methylation in brain. |
| 11 | *D'Addario et al. (2013)* | Animal (rat) | Adulthood | 1 | Alcohol | Amygdala | Candidate | *Pdyn, Pnoc* | Male rats exposed to alcohol for either 1 day, 5 days or 5 days plus 1 withdrawal day. Each group compared to respective water-administered controls (n = 7/group). | No significant association between alcohol exposure and DNA methylation of opioid peptide genes *Pdyn* and *Pnoc* within the amygdala. However, alcohol exposure associated with alterations in specific histone marks. |
| 12 | *Qiang et al. (2014)* | Animal (mouse) | Adulthood | 1 | Alcohol | PFC | Candidate | *Nr2b* | Male eight-week old mice exposed to ethanol vs controls (n = 8 per group). | Chronic intermittent ethanol exposure associated with decreased *Nr2b* methylation (18 CpGs) and increased expression levels in cases s controls. |
| 13 | *Rodrigues et al. (2012)* | Animal (rat) | Adulthood | 1 | Alcohol/opiate /morphine | Nucleus accumbens | Candidate | *Drd2* | Male rats exposed to glucocorticoids in utero vs controls. Within these groups, half exposed to repeated morphine administration in adulthood (4 per group; 16 total). | Repeated morphine exposure associated with increased methylation (and decreased expression) of *Drd2* in rats exposed to glucocorticoids. *Drd2* methylation and expression changes in response to morphine reversed by L-dopa treatment. |
| 14 | *Tammen et al. (2014)* | Animal (mouse) | Adulthood | 1 | Alcohol | Liver | Global | - | Eighteen 18-month-old male mice and twenty 4-month-old mice. | No effect of alcohol consumption on global DNA methylation. Reduced hydroxymethylation observed relative to mice fed a control diet in young, but not old mice. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 15 | *Khachatooria n et al. (2013)* | Animal (rat) | Adulthood | 1 | Alcohol | Liver | Candidate | *24 genes* | Male rats fed ethanol or control diet, with or without SAM supplement for 1 month (n = 3/group). | Compared to controls, ethanol-fed rats showed higher methylation across genes - this increase was non-significant for each individual gene, but significant for average methylation across genes. SAM-treatment prevented ethanol-induced methylation changes. |
| 16 | *Wani et al. (2012)* | Animal (rat) | Adulthood | 1 | Alcohol | Intestine; colon; kidney; pancreas; liver | Candidate | *Fpgc, Ggh, Pcft, Rft* | Male chronically exposed to ethanol for three months vs controls (n = 6/group). | Chronic ethanol feeding associated with tissue- and gene-specific methylation alterations in folate regulating and transporting genes. |
| 17 | *Srinivasan et al. (2014)* | Animal (mouse) | Adulthood | 1 | Alcohol | Pancreas | Candidate | *Slc5a6* | Adult transgenic mice carrying the full-length human SLC5A6 promoter, alcohol-fed vs controls. | Chronic alcohol feeding associated with increased *Slc5a6* promoter methylation, negatively influencing pancreatic biotin uptake. |
| 18 | *Barker et al. (2013)* | Animal (mouse) | Adulthood | 1 | Alcohol | Blood; 9 brain regions | Candidate | *Htr3a* | 15 male ethanol-drinking mice vs 13 water-drinking controls. | Ethanol-drinking was associated with *Htr3a* hypermethylation in blood and hippocampus, but hypomethylation in the dorsomedial and ventromedial PFC. No significant differences in methylation across the other 6 brain regions investigated. |
| 19 | *Tian et al. (2012)* | Animal (mouse) | Adulthood | 1 | Cocaine/ morphine | Nucleus Accumbens; PFC | Global | - | Adult male mice (no sample size or age information specified). | Cocaine but not morphine induced global DNA hypomethylation in the prefrontal cortex (but not in the nucleus accumbens), which was reversible through methionine. |
| 20 | *Nielsen et al. (2012)* | Animal (rat) | Adulthood | 1 | Cocaine | Corpus callosum | Global; candidate | *Mbp,Plp1, Sox10* | 19 male rats: sham rats with 1 (n = 3) or 30 days (n = 4) of forced abstinence; cocaine self-administration rats with 1 (n = 6) or 30 days (n = 6) of forced abstinence. | No difference in global DNA methylation. Candidate genes: reduced mean methylation of *Sox10* relative to controls, especially after 30-day abstinence compared to 1-day abstinence. No differences in *Mpb* or *Plp1* sites. |
| 21 | *Anier et al (2013)* | Animal (mouse) | Adulthood | 1 | Cocaine | Nucleus accumbens; cerebellum | Candidate | *Slc17a7, Cck and Gal* | Male mice (5-6 months old) randomly assigned to one of four conditions (n = 8 per group): (i) saline + saline, (ii) SAM + saline, (iii) saline + cocaine), or (iv) SAM + cocaine. | Repeated SAM pre-treatment modifies cocaine-induced methylation changes in *Slc17a7, Cck* and *Gal* within the nucleus accumbens but not in the cerebellum. |
| 22 | *Pol Bodetto et al. (2013)* | Animal (rat) | Adulthood | 1 | Cocaine | Caudate putamen | Candidate | *Pp2cβ* | Male 8-9 week old rats injected repeatedly (1 time daily x 10 days) with either cocaine or saline (n = 4/group). | Cocaine-exposed rats showed higher *Pp2cβ* promoter methylation compared to controls. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 23 | *Fragou et al. (2013)* | Animal (mouse) | Adulthood | 1 | Cocaine/heroin | Whole brain; liver | Global | - | Eight-week old male mice with either 1) chronic 7-day heroin administration (n = 9 vs 9 controls) or 2) chronic 14-day cocaine administration (n = 9 vs 9 controls). | No differences in either drug group or tissue vs controls. |
| 24 | *Chao et al. (2014)* | Animal (mouse) | Adulthood | 1 | Cocaine/heroin | Whole brain; liver | Global | - | Eight-week old male mice with either 1) chronic 7-day heroin administration (n= 9 vs 9 controls) or 2) chronic 14-day cocaine administration (n = 9 vs 9 controls). | No effects of heroin on methylation or hydroxymethylation in brain or liver. Hydroxymethylation (but not methylation) was reduced in the cocaine group in liver (but not brain tissue). |
| 25 | *Wilhelm-Benartzi et al. (2012)* | Human | Prenatal | 1 | Alcohol | Placenta | Global | - | Mother-infant pairs (n = 184, gender- and maternal-age matched infants). | Mean LINE-1 (but not AluYb8 or global [mean across 27k]) levels differed by maternal alcohol use during pregnancy. |
| 26 | *Azzi et al. (2014)* | Human | Prenatal | 1 | Alcohol | Blood (cord) | Candidate | *ZAC1* | 254 mother-newborn pairs (subsample from EDEN study; mean age = 30). | Pre-pregnancy and prenatal maternal alcohol intake positively correlated with *ZAC1* methylation at birth, which in turn associated with fetal and postnatal weight. |
| 27 | *van der Knaap et al. (2014)* | Human | Adolescence | 1 | Alcohol, cannabis | Blood | Candidate | *COMT* | 463 adolescents (mean age =16, 51% F). | No association between membrane-bound *COMT* promoter methylation and alcohol or cannabis use; significant interaction with *COMT* Val108/158 Met genotype in predicting high-frequent cannabis use. |
| 28 | *Ponomarev et al. (2012)* | Human | Adulthood | 1 | Alcohol | Superior frontal cortex | EWAS | - | Postmortem tissue from 17 alcoholics and 15 matched controls (6% females). DNA methylation available for a subsample of 6 cases and 6 controls. | DNA hypomethylation in the LTR region of 3 retrotransposon gene families: *MLT2A1, THE1B, LTR8.* |
| 29 | *Zhu et al .(2012)* | Human | Adulthood | 1 | Alcohol | Blood | Global | - | Adult alcohol drinkers (n = 717) vs non-drinkers (n = 609); mean age = 62, 14% females. | Lower Alu (but not LINE-1) methylation levels in drinkers vs non-drinkers. |
| 30 | *Zhang et al. (2012)* | Human | Adulthood | 1 | Alcohol | Blood | Candidate | *OPRM1* | 125 cases with alcohol dependence (mean age = 41, 35% F) vs 69 controls (mean age = 39, 51% F) | Higher overall *OPRM1* methylation in cases vs controls. Associations with individual CpG sites (n = 16) did not survive multiple correction. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 31 | *Glahn et al. (2014)* | Human | Adulthood | 3 | Alcohol | Blood | Candidate | *ANP, AVP* | 99 alcohol-dependent patients (mean age = 43) vs 101 controls (mean age = 36). All male. | Significant difference in *AVP* and *ANP* methylation between cases and controls at baseline (day 1). No difference between day 1 and 7 of alcohol withdrawal. *AVP* and *ANP* methylation (1 CpG each) significantly reduced in cases vs controls between day 7 and 14. DNA methylation not significantly associated with serum levels. |
| 32 | *Heberlein et al. (2013)* | Human | Adulthood | 3 | Alcohol | Blood | Candidate | *NGF* | 57 male alcohol-dependent patients during withdrawal. | No significant change in *NGF* methylation and serum levels between day 1 and 7 of alcohol withdrawal. Increase in methylation and decrease in serum levels between day 7 and 14. |
| 33 | *Beach et al (2013)* | Human | Adulthood | 1 | Alcohol, cannabis | Blood | Candidate | *5-HTT* | 155 adult females from the Iowa adoptees sample (mean age = 41). | No significant association between 5-*HTT* methylation and substance use. |
| 34 | *Nieratschker et al. (2014)* | Human | Adulthood | 2 | Alcohol | Blood | Candidate | *DAT* | 100 alcoholic patients (mean age = 47, 20% F) vs 100 matched controls. Repeated DNA methylation available for 85 cases (mean age = 47, 18% F). | No difference in *DAT* methylation between alcoholic patients vs controls; no significant change pre- or post-withdrawal in patients; trend association between higher craving and lower methylation. |
| 35 | *Zhang et al. (2013)* | Human | Adulthood | 1 | Alcohol | Blood | Candidate | *82 genes* | 285 Alcohol dependent cases (mean age = 42, 49% F) vs 249 controls (mean age = 37, 62% F). Replication sample: 49 cases (mean age = 42, 47% F) and 32 controls (mean age 37, 63% F). | Of 384 sites examined (82 genes), one probe in the promoter region of *HTR3A* was significantly associated with European American cases vs controls, after multiple correction (trend association in the replication sample). No significant differences between cases and controls in African Americans. |
| 36 | *Zhang et al. (2013)* | Human | Adulthood | 1 | Alcohol | Liver | Candidate | *UGT1A1* | 46 human liver bank samples (mean age = 41; 46% F). | No association between alcohol history and *UGT1A1* methylation (6 CpGs) in liver. However, alcohol history influenced the relationship between DNA methylation and UGT1A1 protein content and enzymatic activity. |
| 37 | *Yasar et al. (2013)* | Human | Adulthood | 1 | Alcohol | Blood | Candidate | *LEP* | 164 alcohol dependent patients (mean age = 43; 21% F). | Lower LEP promoter methylation at baseline associated with higher serum leptin levels during detoxification (day 7) and stronger alcohol craving in alcohol dependent patients. |
| 38 | *Hillemacher et al. (2015)* | Human | Adulthood | 1 | Alcohol | Saliva | EWAS | - | Community sample of 309 hazardous drinkers (age = 21-55, 31%F). Replication sample: 39 individuals from a | 28 sites were significant after FDR correction, where greater methylation levels were associated with poorer control over drinking. Increased *ALDH1A2* methylation also related to faster increase of breath alcohol levels |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | pharmacological intervention study (mean age = 44; 30% F). DNAm platform: Illumina 27k. | and increased subjective feeling of intoxication. |
| 39 | *Harlaar et al. (2014)* | Human | Adulthood | 1 | Alcohol | Blood | EWAS | - | 63 male alcohol-dependent in-patients (mean age = 44) and 65 age- and sex-matched healthy controls. DNAm platform: Illumina 27k. | 1710 sites were differentially methylated in cases vs controls with $p < 0.005$. Top-ranked sites included genes previously shown to be related to alcohol metabolism (e.g. *ADH1A, ALDH3B2, CYP2A13*). Enriched biological pathways for hypomethylated sites included defence response, response to external stimulus and immune system process, while hypermethylated sites were enriched for mitosis and signal transduction. |
| 40 | *Philibert et al. (2012)* | Human | Adulthood | 1 | Alcohol | Blood | EWAS | - | 165 age-matched females: (i) 40 abstinent (mean age = 47), (ii) 47mild, (iii) 50 moderate, and (iv) 28 heavy alcohol users. DNAm platform: Illumina 450k. | No differences pre vs post treatment. *ABR* differentially methylated in heavy/moderate alcohol users vs abstinent patients. |
| 41 | *Philibert et al. (2014)* | Human | Adulthood | 1 | Alcohol | Blood | EWAS | - | 10 alcohol-dependent patients (mean age = 45) and their discordant siblings as controls (mean age = 44). Sex not specified. DNAm platform: Illumina 450k. | 865 hypomethylated and 716 hypermethylated sites in alcohol dependent patients vs controls *(SSTR4* most hypomethylated; *GABRP* most hypermethylated). Enriched pathways related to metabolic function. |
| 42 | *Zhao et al. (2013)* | Human | Adulthood | 2 | Alcohol | Blood | EWAS | - | Two time points within 12-year period; cases (n = 10; mean age = 40; males) differ from age- and sex- matched control (n = 10) in alcohol consumption at time point 2, but not 1. DNAm platform: Illumina 27k. | 200 differentially methylated sites in cases between time points with a majority of sites increasing in methylation over time; sites correlated with alcohol consumption over 12-year period related to genes such as *CKM, PHOX2A, NPDC1, ADCY9.*Enriched pathways included signal transduction, Notch signalling, and p53 network. |
| 43 | *Weng et al. (2014)* | Human | Adulthood | 2 | Alcohol | Blood | EWAS | - | 33 subjects with heavy alcohol consumption (mean age = 45; 24% F) and 33 age- and sex-matched controls; repeated measures for 25 cases before and after 25-day treatment program. DNAm platform: Illumina 450k. | 8636 FDR- corrected differentially methylated sites (56 Bonferroni-corrected). Age, gender and ethnicity not included as covariates. Enriched pathways included apoptosis and GTPase signalling. No observed differences before vs after treatment. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 44 | *Doehring et al. (2013)* | Human | Adulthood | 1 | Opiate | Blood | Global; candidate | *OPRM1* | 85 methadone-substituted former opiate addicts (mean age = 35; 30% F) vs matched healthy controls. Replication sample: 63 opioid-treated pain patients (age > 50; 43% F) vs matched non-opioid treated controls. | Higher *OPRM1* methylation (1 site) and global (LINE-1) methylation in former opiate addicts vs controls. Association with global methylation reproduced in an independent sample of opiate-treated pain patients vs controls. Within this sample, higher global methylation was also positively associated with average daily opioid dose and pain scores. |
| 45 | *Desplats et al. (2014)* | Human | Adulthood | 1 | Meth | Frontal cortex | Global; EWAS | - | HIV-seropositive cases with (n=13) and without (n=14) methamphetamine-abuse (age- and gender-matched). DNAm platform: Illumina 450k. | Increased global methylation in methamphetamine HIV+ users compared to HIV+ non-users; EWAS: enriched pathways included L-DOPA degradation, ERK/MAPK signalling and Dopamine-DARPP32 feedback in cAMP signalling. |